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Multiple Amine Oxidases in Cucumber Seedlings[1]

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ABSTRACT

Cell-free extracts of cucumber (*Cucumis sativus* L. cv. National Pickling) seedlings were found to have amine oxidase activity when assayed with tryptamine as a substrate. Studies of the effect of lowered pH on the extract indicated that this activity was heterogeneous, and three amine oxidases could be separated by ion exchange chromatography. The partially purified enzymes were tested for their activities with several substrates and for their sensitivities to various amine oxidase inhibitors. One of the enzymes may be a monoamine oxidase, although it is inhibited by some diamine oxidase inhibitors. The other two enzymes have properties more characteristic of the diamine oxidases. The possible relationship of the amine oxidases to indoleacetic acid biosynthesis in cucumber seedlings is discussed.

One pathway which has been proposed for the biosynthesis of IAA in higher plants involves the intermediate formation of TNH_2[4] (4). Cucumber hypocotyl segments elongate in response to applied TNH_2 (14), a growth response which is attributable to conversion of the amine in the plant tissue to IAA. That these reactions take place in cucumber seedlings was shown by Sherwin and Purves (15), using radioisotope techniques. They demonstrated, further, that the production of ^{14}C-IAA from ^{14}C-TNH_2 did not depend upon the presence of epiphytic microorganisms. The first reaction in this process is thought to be an oxidative deamination of TNH_2 to IAAld catalyzed by an amine oxidase (3).

The amine oxidases are a heterogeneous class of enzymes (7). Most investigations have dealt with enzymes isolated from mammalian sources because of the importance of the enzymes to the metabolism of the biogenic amines. The properties of the enzymes differ depending on the organism used for a source as well as on the organ chosen for the isolation. Differences exist both in substrate specificities and in the sensitivities of the enzymes to various inhibitors. The class of amine oxidases was originally divided into monoamine and diamine oxidases (23). However, overlaps in the substrates oxidized by the two types of enzymes led to an alternate system of classification based on inhibitor specificity. Some amine oxidases are inhibited by semicarbazide and carbonyl reagents while others are insensitive to these compounds (1).

The study of plant amine oxidation has focused on the characterization of one enzyme—the amine oxidase of pea seedlings (7). The substrates most efficiently oxidized are the short chain aliphatic diamines (5), and the enzyme is strongly inhibited by semicarbazide and carbonyl reagents (9). On the other hand, Werle and Roewer have isolated enzymes from several plant sources that catalyze the oxidation of short chain aliphatic monoamines and which were insensitive to KCN and semicarbazide (19, 20). A number of plant species have also been shown to have a polyamine oxidase (16). Thus, it appears that the same diversity found in animal amine oxidases may also characterize the plant enzymes. This paper will present evidence for the existence of multiple amine oxidases with varying properties in cucumber seedlings. All of the enzymes are able to catalyze the oxidation of TNH_2.

MATERIALS AND METHODS

Plant Material. Seeds of *Cucumis sativus* L. cv. National Pickling (Burpee) were surface-sterilized with 2.5% Clorox and soaked in a distilled water solution of ammonium nitrate (400 mg/l). The seedlings were grown under a 14L 10D cycle at 30 C and were watered every other day with the planting solution.

Preparation of Enzymes. Seven-day-old seedlings were harvested and ground for 1 min in a commercial 1-gal Waring Blendor using a 1:1 ratio (w/v) of 50 mM sodium phosphate buffer, pH 8. The homogenate was squeezed through eight layers of cheesecloth and centrifuged for 40 min at 16,000*g*. The supernatant fluid was filtered through glass wool to remove solidified lipids, and the filtrate was used either directly as the enzyme preparation or as the starting material for further purification steps.

Acid precipitation was carried out on the crude extract to purify amine oxidases from inactive material. Acetic acid (0.5 N) was added dropwise to a large volume of the enzyme preparation with vigorous stirring until the pH of the solution reached 4.6. Precipitated material was removed by centrifugation at 16,000*g* for 20 min. Ammonium sulfate fractionation of the supernatant fluid was performed at 0 C. Powdered ammonium sulfate was added slowly with stirring to the enzyme solution to a level of 30% saturation. This was followed by a 20-min period of stirring to allow equilibration. The suspension was centrifuged and the pellets were discarded. The supernatant fluid was brought to 60% saturation, and the pellet from subsequent centrifugation was suspended in a small amount of 50 mM sodium phosphate buffer, pH 7.5, and dialyzed against 20 volumes (two changes) of the same buffer overnight.

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[4] Abbreviations: TNH_2: tryptamine; MAO: monoamine oxidase; DAO: diamine oxidase; IAAld: indole-3-acetaldehyde; DEAE: diethylaminoethyl.

The 30 to 60% ammonium sulfate fractions from two extractions (4 kg fresh weight of tissue) were pooled and poured through a 5 × 35 cm column of DEAE-cellulose (Schleicher and Schuell) equilibrated with 20 mM sodium phosphate buffer, pH 7.5. Material not binding to the column was saved, concentrated by ammonium sulfate precipitation (70% saturation), dialyzed, and stored frozen in 20% sucrose. The column was washed with 35 mM sodium phosphate buffer, pH 7.5, and bound substances were eluted with a linear salt gradient (0 to 0.5 M NaCl in 35 mM buffer). The total volume of the gradient was 2 liters, and 11-ml fractions were collected. Fractions containing TNH_2 oxidase activity equal to half or more of that of the peak tubes were pooled, and the resulting solutions were dialyzed overnight against 20 mM sodium phosphate buffer, pH 7.5. The first amine oxidase to elute from the column (see "Results") was bound to the top of a 3 × 30 cm DEAE-cellulose column and was eluted with a linear salt gradient (1 liter, 0 to 0.15 M NaCl in 35 mM sodium phosphate buffer, pH 7.5), collecting 5-ml fractions. The second amine oxidase from the first DEAE-cellulose step was similarly rechromatographed on DEAE-cellulose, except that the column was first washed with 50 mM NaCl in 35 mM buffer before eluting the enzyme with a linear salt gradient (0.05 M to 0.25 M NaCl in 35 mM buffer).

The material that did not bind to the first DEAE-cellulose column was dialyzed against 35 mM sodium phosphate buffer, pH 6, and then was bound to the top of a 5 × 35 cm column of cellulose phosphate (Schleicher and Schuell) equilibrated with the same buffer. Bound substances were eluted with a linear salt gradient (2 liters, 0 to 0.5 M NaCl in 35 mM buffer), and 10 ml fractions were collected.

Each of the enzyme preparations obtained by ion exchange chromatography was concentrated by ammonium sulfate precipitation (70% saturation), redissolved in 4 ml of 20 mM sodium phosphate buffer, pH 7.5, and dialyzed against 50 mM buffer at the same pH to remove excess ammonium sulfate. The concentrated enzymes were subjected individually to gel filtration through a 2.5 × 36 cm column of Sephadex G-200 (Pharmacia) equilibrated with 50 mM sodium phosphate buffer, pH 7.5. The column was calibrated with nonenzymic protein mol wt markers (Mann) to allow an estimation of the mol wt of the enzymes. Material was eluted from the column by gravity flow using a constant pressure head, and 3-ml fractions were collected.

Reduction of the Product of TNH_2 Oxidation with Sodium Borohydride. An aliquot of the enzyme preparation (0.3 ml) was incubated with 0.1 ml of 2-^{14}C-TNH_2 bisuccinate (0.1 μCi) (New England Nuclear) for 4 hr. Approximately 5 mg of sodium borohydride was added to the reaction mixtures following the addition of carrier IAAld. The reduction was allowed to proceed for 1 hr, the mixture was acidified and extracted with ether, and the ether phase was analyzed by TLC (ether-hexane, 7:1) on 250 μm silica gel plates (Brinkman). Radioactivity was determined either by strip scanning, using a Nuclear Chicago Actigraph II, or by liquid scintillation counting of areas scraped from the plate.

Assay Procedures. TNH_2 oxidase was assayed by a modification of the procedure developed by Wurtman and Axelrod (22). The reaction mixture contained 0.1 ml of the enzyme preparation, 0.2 ml of buffer or other additions, and 0.1 ml of ^{14}C-TNH_2 bisuccinate (0.05 μCi). In assaying fractions from purification steps, no carrier TNH_2 was added, to avoid dilution of the label. In other studies, nonradioactive TNH_2 was added to give the indicated concentrations. To stop the reactions, 0.25 ml of 2 N HCl was added to each reaction mixture followed by 5 ml of a scintillation counting solution containing 4 g/l of PPO and 50 mg/l of POPOP in toluene. The tubes were shaken vigorously using a Vortex mixer for 20 sec, and the phases were separated by centrifugation. A 4-ml aliquot of the organic layer was removed for scintillation counting using a Beckman LS-150 counter. Acidic and neutral compounds are extracted into the organic phase in this procedure, leaving the basic substrate in the aqueous layer.

Putrescine oxidase was assayed according to Holmstedt *et al.* (6). The enzyme (0.4 ml) was incubated with 0.5 ml of 5 mM *o*-aminobenzaldehyde in 50 mM sodium phosphate buffer, pH 7.4, and 0.1 ml of 0.1 M putrescine. The product of the reaction, Δ^1-pyrroline, reacts with *o*-aminobenzaldehyde to give a product which absorbs light at 430 nm. The reactions were stopped by the addition of 0.2 ml of 10% trichloroacetic acid to the mixtures, and the samples were centrifuged to remove precipitated material. The absorbances at 430 nm of the samples were read against a blank containing all ingredients except putrescine.

Benzylamine oxidase was assayed by a modification of the method of Tabor *et al.* (17). Reaction mixtures contained 0.3 ml of enzyme, 0.4 ml of 50 mM sodium phosphate buffer, pH 7.5, and 0.3 ml of 10 mM benzylamine. The reactions were stopped with 0.2 ml of 10% trichloroacetic acid, and the precipitated material was removed by centrifugation. The absorbance of the supernatant fluid at 250 nm was determined, using as a reagent blank a reaction mixture which had been acidified immediately after addition of the substrate.

For kinetic studies involving different substrates, the peroxidase-coupled fluorometric assay of McGowan and Muir (13) was applied. The assay mixture contained 1.2 ml of buffer, 0.2 ml of substrate, 0.4 ml of enzyme, 0.1 ml of horseradish peroxidase (1 mg/ml), and 0.1 ml of 0.1 mM scopoletin, a fluorescent peroxidase substrate. The reaction was monitored by following the disappearance of scopoletin fluorescence using a Perkin-Elmer MPF-2A fluorescence spectrophotometer. The excitation and emission wavelengths were 370 and 465 nm, respectively.

Protein was determined by the Lowry method (8), or, in the case of column eluates, the absorbance at 280 nm was taken as a measure of protein concentration.

RESULTS

Acid Precipitation of Amine Oxidases. Cell-free extracts of cucumber seedlings were found to have amine oxidase activity when assayed with ^{14}C-TNH_2. As a step toward developing a purification procedure, a study of the effect of lowered pH on the enzyme activity was performed. Aliquots (20 ml) of the cell-free extract (initial pH 6.9) were adjusted to various pH values using 0.4 N HCl added dropwise with manual mixing. The solutions were centrifuged, the pellets were discarded, and the supernatant solutions were dialyzed overnight against 50 mM sodium phosphate buffer, pH 7.4. The amine oxidase activities of the different fractions were assayed using both ^{14}C-TNH_2 and putrescine as substrates. As shown in Figure 1, the TNH_2 oxidase and putrescine oxidase activities were lost from the extract at different pH levels. These results point to the existence of more than one amine oxidase in cell-free preparations of cucumber seedlings.

In performing the acid precipitation on a preparative scale, using 0.5 N acetic acid, we found that no loss of TNH_2 activity occurred, perhaps because of the more gradual lowering of the pH. Since these preparations also retained putrescine oxidase activity, extracts which had been adjusted to pH 4.6 with acetic acid and centrifuged to remove precipitated protein were used as the starting material for further purification of all the enzymes.

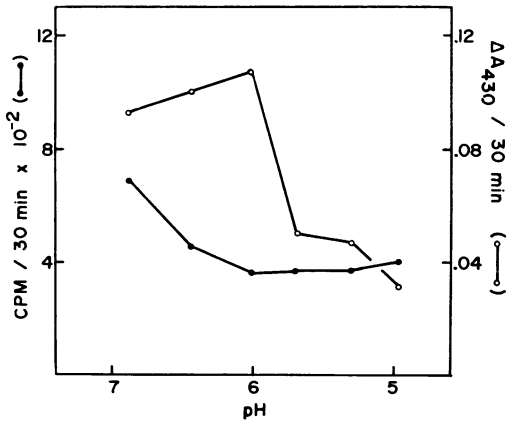

FIG. 1. Acid precipitation of amine oxidases. The enzyme preparation was adjusted to the pH indicated, and then it was adjusted to pH 7.4 by dialysis. ●: TNH_2 oxidase assayed by radioisotope method; ○: putrescine oxidase.

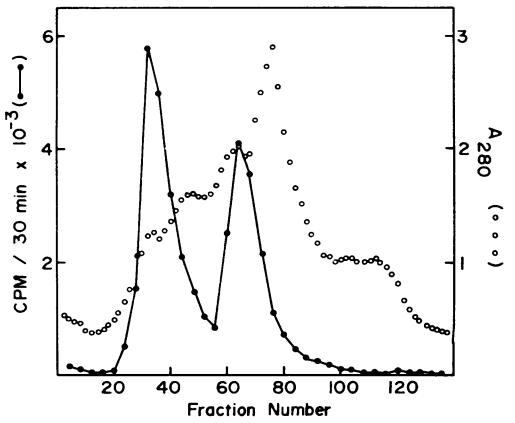

FIG. 2. DEAE-cellulose chromatography of acidic amine oxidases. TNH_2 oxidase was assayed by the radioisotope method, and the absorbance at 280 nm was a measure of protein concentration.

Purification of Enzymes. To obtain a more direct demonstration of the heterogeneity of the cucumber amine oxidase activity, a separation of the enzymes was attempted. The enzyme preparation, partially purified by acid precipitation and ammonium sulfate fractionation, was passed through a column of DEAE-cellulose. Material which did not bind was passed through a column of cellulose phosphate. Bound material was eluted from both columns with linear salt gradients and the fractions were assayed for amine oxidase activity. As shown in Figures 2 and 3, there were two amine oxidases which bound to DEAE-cellulose and at least one which bound to cellulose phosphate. The peak for the latter enzyme was broad, and it had a shoulder, indicating that this fraction still might have been heterogeneous. For the studies reported below, however, it was treated as a single enzyme. For the sake of brevity, the first and second enzymes to elute from the DEAE-cellulose column are referred to as AO-1 and AO-2, respectively, while the enzyme preparation obtained from cellulose phosphate chromatography is called AO-3.

The final step in the isolation was a gel filtration of the enzymes through Sephadex G-200. Approximate mol wt of the three enzymes were determined by comparing their elution volumes from this column to those of ovalbumin (M = 45,000), BSA (M = 67,000), human gamma globulins (M = 160,000), and horse apo-ferritin (M = 480,000). The results are presented in Figure 4. The three enzymes have very similar mol wt. AO-2 and AO-3 both have a mol wt of about 100,000, while AO-1 is somewhat smaller, having a mol wt of approximately 87,000.

The purification procedure used in these studies results in approximately a 150-fold purification of all three enzymes relative to the initial crude homogenate. Table I summarizes the procedure for the case of AO-2. The purification achieved for each protein is actually higher, since the activity in the crude homogenate was the sum of the three enzymes. More important for the purposes of this study, however, was the degree of separation achieved for each enzyme from the other amine oxidases. AO-1 was obtained free from contamination by the other enzymes, but it was not possible to prepare AO-2 free of AO-1. After an examination of the elution profiles for the enzymes from DEAE-cellulose chromatography, it seemed reasonable to assume that the bulk of TNH_2 oxidation observed in AO-2 preparations was actually due to AO-2 and not to contaminating AO-1. In addition, preliminary studies of the dependence of the enzyme reactions on pH showed that there was a greater relative activity of AO-2 at pH 7. Therefore, subsequent studies of AO-2 were carried out at this pH, using TNH_2 as a substrate. AO-3 was treated as a single enzyme although, as discussed above, this preparation still might have been heterogeneous.

Proofs of Product. The cucumber enzymes were isolated us-

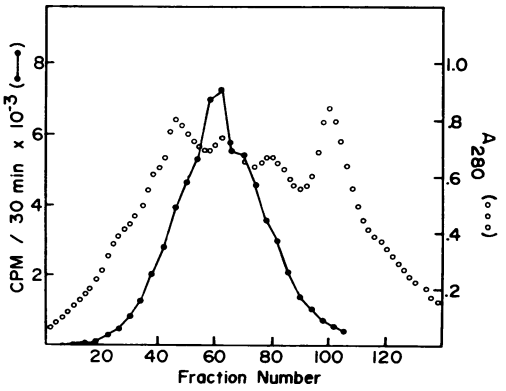

FIG. 3. Cellulose phosphate chromatography of basic amine oxidase. Assay methods were as in Figure 2.

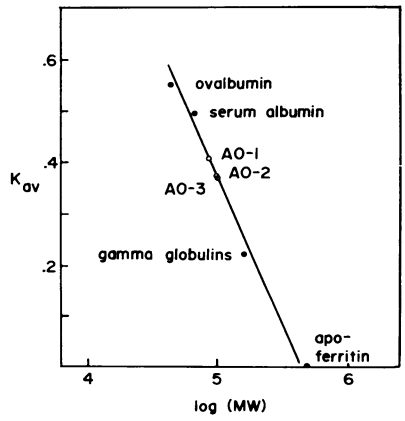

FIG. 4. Mol wt determinations for the cucumber amine oxidases. The elution of the enzymes from Sephadex G-200 was compared to that of nonenzymic proteins of known mol wt. $K_{av} = (V_e - V_o)/(V_T - V_o)$, where V_e is the elution volume of the protein, V_o is the void volume of the column, and V_T is the total bed volume.

Table I. *Purification of AO-2*

Aliquots of fractions from the different purification steps were diluted to give volumes corresponding to the volume of the initial crude homogenate. These were assayed with the radioisotope assay.

	Total Protein	Units[1]	Yield	Specific Activity	Fold Purified
	mg		*%*	*units/mg protein*	
Crude extract	11,900	3950	100	0.33	1
pH 4.6 supernatant	1,935	5280	134	2.99	9
30 to 60% $(NH_4)_2SO_4$ fraction	1,170	4890	124	4.2	12.6
First DEAE step	202	3880	97	17.4	52.6
Second DEAE step	72.5	3390	86	46.5	141
$(NH_4)_2SO_4$ concentration	64	2750	70	35	106
Sephadex G-200	53	2750	70	52.2	158

[1] Amount of enzyme required for the conversion of 1000 cpm of radioactive TNH_2 to product.

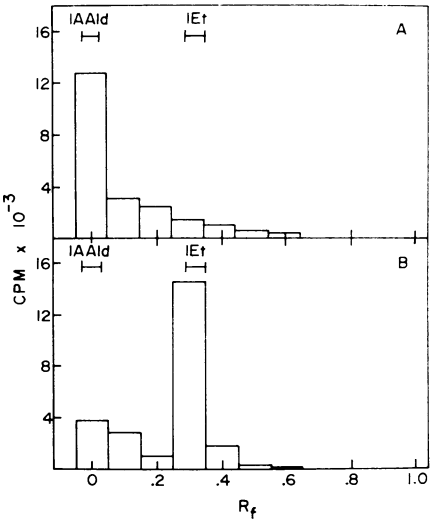

FIG. 5. TLC of the reaction product of AO-2 acting on ^{14}C-TNH_2 (A), and the reaction product reduced with sodium borohydride (B). Radioactivity was determined by scintillation counting of areas scraped from the plate.

ing an assay that monitors the conversion of ^{14}C-TNH_2 to either a neutral or an acidic radioactive product. Clarke and Mann (2) showed that pea seedling amine oxidase catalyzed the formation of IAAld from TNH_2. This also appears to be the case with the cucumber enzymes. The reaction product from the incubation of AO-2 with ^{14}C-TNH_2 was reduced with sodium borohydride, a reducing agent for carbonyl compounds. The product of the reduction reaction was analyzed by TLC, and distribution of radioactivity on the plate is shown in Figure 5. The bulk of radioactivity in samples not treated with borohydride co-chromatographs with authentic IAAld. Treatment with borohydride results in the reduction of this peak and the appearance of a new peak at the R_F of indole-3-ethanol. These results indicate that IAAld is the product of the reaction, with TNH_2 catalyzed by AO-2.

Similar results were obtained from the same experiment with AO-3, and they are shown in Figure 6. In addition to the peak of radioactivity corresponding to IAAld, there is a second radioactive compound at a slightly higher R_F which was not affected by the borohydride treatment, however. Neither the identity of the compound nor the mechanism by which it is formed are known at present. The enzyme preparations used in these studies are not pure, however, and the second compound may be the product of further metabolism of IAAld catalyzed by some other enzyme. Alternatively, the substance may be an IAAld breakdown product which is formed nonenzymically in the assay procedure. In either case, its formation seems to depend on amine oxidase activity, since the conversion of ^{14}C-TNH_2 to labeled acidic or neutral products by this enzyme preparation may be strongly inhibited by amine oxidase inhibitors (see below).

When the same experiment was performed using AO-1, there was no formation of ^{14}C-indole-3-ethanol by sodium borohydride reduction of the reaction mixture. The bulk of the radioactivity in either treated or untreated samples moved with the same R_F as the second compound observed in the experiment with AO-3. As with AO-3, some amine oxidase inhibitors completely inhibited the conversion of ^{14}C-TNH_2 to labeled products. It seems likely, then, that the reaction of TNH_2 observed in this assay is catalyzed by an amine oxidase as well and that, by analogy with other amine oxidases, the initial product formed is IAAld. Putrescine and benzylamine are also acted on by AO-1, and the products formed have the properties of the corresponding aldehydes.

Effects of Inhibitors on the Enzymes. Amine oxidases are subject to inhibition by a variety of compounds (7). The metalloenzymes are inhibited by chelating agents, and the differential sensitivities of the enzymes to carbonyl reagents have served as the basis for a system of classification. Monoamine oxidases tend to be insensitive to these compounds while diamine oxidases are inhibited by them. Since inhibitor sensitivity has been a useful criterion for distinguishing amine oxidases, the effects of several inhibitors on the three cucumber enzymes were determined (Table II). In these studies, as well as those of the substrate specificities of the enzymes, AO-1 and AO-3 were assayed at their pH optima (8.7 and 7.5, respectively) determined in separate experiments, and AO-2 was assayed at pH 7, as discussed above. All three of the cucumber amine oxidases were inhibited by tranylcypromine, a MAO inhibitor. Another compound, 1-Octanol, which is thought to be selective for MAO, inhibited AO-1 and AO-2 more strongly than AO-3. Iproniazid, an inhibitor of both MAO and DAO, inhibits AO-3

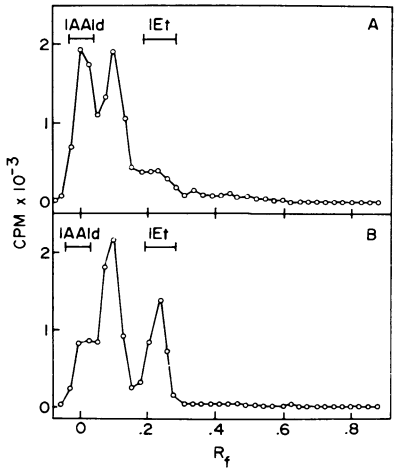

FIG. 6. TLC of the reaction product of AO-3 acting on ^{14}C-TNH_2 (A), and the reaction product reduced with sodium borohydride (B). Radioactivity was determined by strip scanning.

Table II. *Effects of Inhibitors on AO-1, AO-2, and AO-3*

The inhibitors were supplied at the indicated concentrations. The enzymes were assayed by the radioisotope method using 0.2 mM TNH_2. The buffers were 50 mM sodium borate, pH 8.7 (AO-1), 50 mM sodium phosphate, pH 7.0 (AO-2), and 50 mM sodium phosphate, pH 7.5 (AO-3).

	Concentration	Inhibition		
		AO-1	AO-2	AO-3
		%		
Tranylcypromine	0.1 mM	65.3	85.7	100
	1.0 mM	97.1	99.8	100
Aminoguanidine	1.0 μM	62.2	10.1	96.8
	10 μM	100	15.1	100
KCN	0.1 mM	33.0		60.2
	1.0 mM	83.1	0.8	77.6
Iproniazid	10 μM	16.7	3.3	57.3
	100 μM	63.3	64.0	90.0
Isoniazid	10 μM	7.7	34.4	30.5
	100 μM	19.3		76.8
Semicarbazide	1.0 μM	35.3	50.2	88.9
	10 μM	98.5	92.0	100
1-Octanol	5% saturated	43.3	24.5	12.3
	25% saturated	69.1	64.7	17.5
Iodoacetate	10 mM	4.0	1.6	0.9
Sodium diethyldithiocarbamate	0.1 mM	90.7		37.5
	1.0 mM	99.2	25.9	75.6
α,α′-Dipyridyl	0.1 mM	92.3		99.5
	1.0 mM	99.1	97.8	100
Cuprizone	25% saturated (<0.1 mM)	95.2		98.0
Neocuproine	0.1 mM	3.3		14.7

more strongly than AO-1 or AO-2. This result is hard to interpret, however, since many investigators feel that iproniazid is hydrolyzed *in vivo* or in crude homogenates to form isopropylhydrazine and nicotinic acid, and that it is the hydrazine rather than the parent compound that actually inhibits the enzyme (7). Thus, different sensitivities of the cucumber enzymes to this inhibitor may only reflect different hydrolase activities in the enzyme preparations.

AO-1 and AO-3 are inhibited much more strongly by the DAO inhibitors aminoguanidine and KCN than is AO-2. This raises the possibility that AO-2 might be a MAO similar to those reported by Werle and Roewer (19, 20). All of the enzymes, including AO-2, are inhibited by semicarbazide, however; and, in addition, AO-2 is also inhibited by isoniazid, another DAO inhibitor.

Studies with other inhibitors were directed toward demonstrating the participation of specific components of the enzyme in the reaction. None of the enzymes was significantly inhibited by iodoacetate, indicating a lack of involvement of sulfhydryl groups in the enzyme reaction. Pea seedling amine oxidase is reported to contain Cu^{2+} which is required for activity (5). Studies of the effects of chelating agents pointed to the presence of a required metal ion in the cucumber enzymes as well. All of the enzymes were inhibited by sodium diethyldithiocarbamate, a compound which shows some specificity for copper, although AO-1 and AO-3 were inhibited more strongly than AO-2. α,α′-Dipyridyl was also a strong inhibitor of the enzymes. The question of the identity of the metal ion was pursued further with AO-1 and AO-3. Both enzymes were inhibited more strongly by the cupric chelator cuprizone than they were by the cuprous chelator neocuproine, suggesting that AO-1 and AO-3, like the pea seedling enzyme, may be Cu^{2+} enzymes.

Substrate Specificities of the Enzymes. A further comparison of the cucumber amine oxidases was obtained in a study of the substrate specificities of the enzymes. As noted above, AO-2 was not obtained free of contamination by AO-1, precluding a detailed study of the substrate preferences of this enzyme. However, some indication that AO-1 and AO-2 catalyze the oxidation of different amines to different extents is given by the results shown in Figure 7. The distributions of putrescine, benzylamine, and TNH_2 oxidase activities in fractions from the first DEAE-cellulose chromatographic step were determined. There was somewhat less TNH_2 oxidase activity in the AO-2 peak than there was in the AO-1 fractions, the benzylamine oxidase activity of AO-2 was much lower than that of AO-1, and all of the putrescine oxidase activity in preparations of AO-2 can be ascribed to the contaminating tail of the AO-1 peak. It appears, then, that AO-2 may have the substrate specificity of a monoamine oxidase.

Kinetic experiments were performed using the peroxidase-coupled fluorometric assay to determine the substrate specificities of AO-1 and AO-3. Michaelis constants and maximum velocities, relative to that obtained with TNH_2, for several substrates are shown in Table III. The studies reported here involved only a small number of determinations. The values given for the constants are subject to further refinement, therefore,

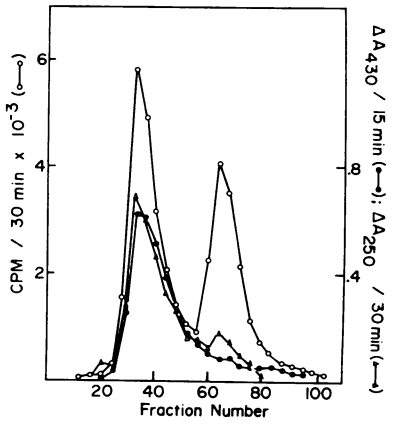

FIG. 7. DEAE-cellulose chromatography of acidic amine oxidases. ○: TNH_2 oxidase; △: benzylamine oxidase; ●: putrescine oxidase.

Table III. *Substrate Specificities of AO-1 and AO-3*

The enzymes were assayed with the different substrates using the peroxidase-coupled assay. Kinetic constants were determined from double-reciprocal plots of the data from experiments in which the concentration of substrate was varied. Maximum velocities are expressed relative to the maximum velocity obtained with TNH_2 (AO-1: 2.6 nmoles/min; AO-3: 0.75 nmoles/min). The buffers used were as described in Table II.

	AO-1		AO-3	
	K_m	V_{max}	K_m	V_{max}
	μM		μM	
Putrescine	4.8	43.8	10	2.2
Spermidine	14	21.0	40	1.0
Benzylamine	7.9	4.2	9.3	0.5
Butylamine	18	1.3	24	0.9
Tryptamine	180	1.0	120	1.0

and are intended to provide a rough comparison of the enzymes.

Both AO-1 and AO-3 are able to catalyze the oxidation of a variety of amines as indicated by their activities with representative compounds. A comparison of the kinetic constants for the amines tested shows that AO-1 and AO-3 are similar in their affinities for the various substrates, but that the relative maximum velocities obtained with these compounds differ markedly for the two enzymes. For example, the clearly preferred substrate for AO-1 was putrescine. Although putrescine was also the substrate most rapidly oxidized in AO-3 preparations, the maximum rate of putrescine oxidation is only about twice that of TNH_2 oxidation. AO-1 catalyzes the reaction with putrescine at a rate over 40 times faster than that with TNH_2. Similar comparisons of AO-1 and AO-3 can be made using the results from the other substrates, and these also indicate that the two enzymes differ in their catalytic properties.

DISCUSSION

To our knowledge, this is the first demonstration of multiple amine oxidases occurring in a single plant species. Although Werle and Hartung (18) proposed that pea seedlings contain separate enzymes for the oxidation of histamine and short chain diamines, subsequent studies in the same laboratory produced indirect evidence that the two activities were properties of a single protein (21). Studies of purified pea seedling amine oxidase have shown that both types of amines are in fact oxidized by the single enzyme (5). In the work reported here, amine oxidase activity was detected using a sensitive radioisotope assay which permitted the detection of low levels of the enzymes. Application of this technique to other plant species may show that the multiplicity of amine oxidases in cucumber seedlings is not an isolated phenomenon.

AO-1 was the enzyme in these experiments which most closely resembled the pea seedling amine oxidase. Both enzymes oxidize the short chain aliphatic diamines at a much faster rate than they do TNH_2 or the aliphatic monoamines. Both are inhibited by semicarbazide and carbonyl reagents. Both enzymes appear to be Cu^{2+} proteins, based on inhibitor studies and, for the case of the pea enzyme, direct chemical analysis (10). It is unlikely that the two enzymes are homologous proteins, however. The pea enzyme is a basic protein while AO-1 is acidic. The mol wt of AO-1 was estimated to be approximately 87,000. Using electron microscopy, Hill and Mann (5) judged that the mol wt of the pea enzyme was 96,000. However, McGowan and Muir (13) determined the mol wt of the pea enzyme by sedimentation equilibrium analysis and reported a value of approximately 184,000. This value is comparable to those reported for porcine plasma and bovine plasma amine oxidases, which are 195,000 and 170,000, respectively (7). McGowan and Muir (13) suggested that the lower value reported by Hill and Mann (5) was the result of subunit dissociation during the procedures used for electron microscopy. This would not be the case with AO-1, however. Finally, the substrate specificities of the two enzymes, although similar, are not exactly the same, and the *Km* values determined with several substrates are lower for AO-1 than those reported for the pea enzyme (13).

AO-3 was, in some ways, similar to AO-1. In the inhibitor studies, both enzymes behaved as Cu^{2+} proteins, and both showed similar affinities for the amines used in the study of substrate specificity. In terms of maximum velocities, however, AO-3 was unusual in that there was no clearly favored substrate as there was with AO-1. Some differences also exist in the sensitivities of the enzymes to various inhibitors, AO-3 being less sensitive to octanol and more sensitive to isoniazid than AO-1. In this respect, AO-3 may resemble the mammalian diamine oxidases more closely than does AO-1. The generalizations concerning inhibitor specificity developed for the mammalian enzymes may not be directly applicable to plant amine oxidases, however. Yet both enzymes fall into the class of amine oxidases inhibited by semicarbazide and carbonyl reagents.

From the preliminary study with different amines, AO-2 appears to have the substrate specificity of a MAO. The enzyme was relatively insensitive to the DAO inhibitors aminoguanidine and KCN, but it was inhibited by semicarbazide and isoniazid. McEwen (11, 12) has isolated a somewhat similar enzyme from human plasma. The plasma amine oxidase oxidized benzylamine most efficiently while having little activity with putrescine. It was inhibited by semicarbazide, isoniazid, and by the MAO inhibitor octanol. Before a detailed characterization of AO-2 will be possible, however, methods will need to be developed to free the enzyme preparation from contamination by AO-1.

The occurrence of an amine oxidase in cucumber seedlings was suggested by the finding that applied TNH_2 is converted to IAA in hypocotyl segments (15). The isolation of a specific TNH_2 oxidase from these plants would have been evidence in favor of the same pathway being used in normal auxin biosynthesis, but this result was not obtained. Although further study of AO-2 is required for an adequate description of its substrate specificity, the enzyme is active with at least benzylamine, in addition to TNH_2. AO-1 and AO-3 catalyze the reaction with a number of amines, and TNH_2 is not the favored substrate of either enzyme. This ability of the enzymes to work with a variety of amines does not automatically rule out a role for them in IAA biosynthesis, however. The specificities of the mammalian enzymes are similarly broad, and they are thought to catalyze reactions in a number of metabolic pathways (7). Thus, the isolation of the cucumber amine oxidases demonstrates the molecular basis for the ability of tissue segments to respond to exogenous TNH_2, but other evidence is needed to determine their relevance to normal auxin biosynthesis.

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